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EVALUATION OF ANTIBACTERIAL POTENTIAL OF ARTEMISININ EXTRACTS OF *ARTEMISIA ANNUA IN VIVO* AND *IN VITRO*

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ABSTRACT

To ensure universal health care, the World Health Organization recognized the significance of complementary and alternative medicines (CAM) and recommended the use of natural herbs and plants bearing therapeutic potential and fewer adverse effects. Therefore, *Artemisia annua* herb was evaluated for its antibacterial potential and therapeutic efficacy against *Staphylococcus aureus*, Streptococcus and *Escherichia coli* both *in vitro* and *in vivo*. Artemisinin was extracted from *Artemisia annua* by chemical treatment. Subsequently, the culture sensitivity tests were performed on MHA by disk diffusion method to determine the antibacterial potential of the Artemisinin extracts against the test bacteria (*in vitro* phase). The results of this *in vitro* trial revealed that the test bacteria *Staphylococcus aureus* and Streptococcus were significantly sensitive to Artemisinin extracts and showed a diameter of 27.7 and 22.3mm of the bacteriostatic zone, respectively, while the *Escherichia coli* was moderately sensitive to the Artemisinin extracts with the bacteriostatic zone of 12.9mm. During the 2nd phase of the study *(in vivo* trial), 20 rabbits were maintained which were infected with *S. aureus* and were successfully treated with varying concentrations of the Artemisinin extracts @ 1 mg/ml, 2 mg/ml and 5 mg/ml in DMSO and were recovered. Similarly, rabbits infected with Streptococcus were also successfully treated and recovered. Thereafter, rabbits infected with *E. coli* were treated with Artemisinin, and out of 15 rabbits in three test groups, 03 rabbits died while the others were recovered. Hence, as per findings of this study, Artemisinin extracts were recommended against *Staphylococcus aureus* and Streptococcus infections.

Keywords: Artemisinin, alternative medicines, antibacterial potential, Streptococcus infections.

INTRODUCTION

The research and re-evaluation of herbal and traditional plants for their medicinal and therapeutic potential has been increasing worldwide because plant remedies are likely to have fewer adverse effects than synthetic chemical substances used for therapeutic purposes (Kim et al., 2015). World Health Organization recognizes the role of the CAM (Complementary & Alternative Medicine) of demonstrated safety, verified quality, and effectiveness in ensuring universal access to healthcare. While in duration from 2014 to 2023, the traditional medicine strategy of WHO focused the harness the potential contribution of CAM pertaining to healthcare. CAM or medicines derived from herbs or plants are the potential CAM therapies for therapeutic objectives (WHO, 2014). Thus, natural plants can serve as an important source of novel antibacterial agents, worldwide (Tajehmiri et al., 2014).

Artemisia annua is a native herb to Asia, and is in practice in traditional Asian medicine for centuries, regarding the treatment and impediment of pyrexia and malaria. Various compounds are extracted

from the test herb *Artemisia annua* including aliphatic alkanes, coumarins, Artemisinin, flavonoids, phenolics, purines, sesqui-terpenoids, steroids, tri-terpenoids (Bhakuni et al., 2001). Artemisinin is the essential component of *Artemisia annua L.* chemically composed of $C_{15}H_{22}O_{15}$ (Empirical formula). It was discovered by Tu Youyou in 1972 and she was a Nobel Prize recipient in the field of Medicine, in 2015. Artemisinin is widely used as an antibacterial, antimalarial, antitumor, antifungal, antioxidant, antiviral, antileishmanial and anti-inflammatory agent (Efferth, 2007; Konkimalla et al., 2008; Tu, 1999). It is also used as an acaricidal agent against *Rhiphicephalus microplus* ticks. It also has effectiveness against Schistosomiasis and gastrointestinal nematodes, in terms of reduction in the eggs per gram of feces (Chagas et al., 2011).

The essential oils extracted from the Artemisia herb have been extensively used as folk medicine characterized by antispasmodic, antipyretic, anti-inflammatory, and abortifacient properties (Zarga et al., 1995). These essential oils also serve as chief ingredients in soap and detergents, perfumes, cosmetics as well as in aromatherapy. The aerial parts of the *Artemisia annua* plant are also used extensively in Iranian traditional medicine, for curing hypoglycemic, hypolipidaemic, anti-inflammatory and diuretic conditions (Kulkarni, 2002).

Therefore, this study was designed to investigate and compare the efficacy of Artemisinin extracts in vitro, on culture sensitivity tests with commercially used antibiotics and in vivo antibacterial effect of Artemisinin in the rabbit models post bacterial challenge with *S. aureus*, Streptococcus and *E. coli*. And it was found from the results that Artemisinin extracts had significant antibacterial effects @ 1mg/ml MIC (Minimum Inhibitory Concentration), both *in vitro* and *in vivo* against the test bacteria *Staphylococcus aureus*, and Streptococcus, while, *Escherichia coli* was moderately sensitive to Artemisinin extracts.

MATERIALS AND METHODS

Study Area and Period

The study was carried out at Veterinary Research and Disease Investigation Center (VR&DIC), Dera Ismail Khan, Department of Livestock and Dairy Development (Research), Khyber Pakhtunkhwa, Pakistan, during the year 2020-21 and 2021-22.

Plant Material

The fresh plant material of *Artemisia annua* was procured from the local nurseries of District Dera Ismail Khan. The leaves of the plant specimen were washed with tap water to remove the dust and debris. Then the collected plant materials were dried under the shade at 25°C and then mashed with the help of a mortar and pestle (Figure 1).

Figure 1: Weighing the dried leaves of *Artemesia annua* herb.

Production of Artemisinin

About 100 g of plant material of *Artemisia annua* was soaked in 1 Liter of Chloroform (Merck) as a solvent for a fortnight and was agitated twice a day and finally filtered. Then the filtrate was evaporated to acquire the gummy residue (Figure 2) using a rotary evaporator and

the extracts were stored in a sterile glass bottle at room temperature, at STP (Javid et al., 2015).

Artemisinin is soluble in organic solvents but is unstable in the form of an aqueous solution. Therefore, a fresh solution of Artemisinin was prepared from the stock solution on each day required in the experiment @1mg/ml in dimethyl sulfoxide (DMSO, Merck).

Figure 2: The gummy residue of Artemisinin extracts after evaporation of the chloroform.

In Vitro Evaluation of Artemisinin

The bacteria including *Staphylococcus aureus, Escherichia coli* and Streptococcus were cultured in the laboratory facility of the VR&DIC, Dera Ismail Khan from various samples of animal origin including milk, feces, intestinal contents of the poultry, *etc*. The filter paper disks were prepared and impregnated with Artemisinin dissolved in organic solvents DMSO and were used to compare with the 06 commercial antibiotic disks *viz* amoxicillin, enrofloxacin, doxycycline, tetracycline, ciprofloxacin and oxytetracycline in the culture sensitivity test.

For the isolation and cultivation of the bacteria, the following media were used.

Media Preparation

The media required was calculated as per need. For example; 25-30ml agar was required for a single large plate and this amount was mathematically calculated for the number of plates prepared. The media was reconstituted, as per instructions of the manufacturer, e.g. 111g of MSA was dissolved in 1Liter of distilled water. The media was stirred for proper mixing of the media powder in distilled water and was heated to boiling for complete dissolution. The beaker was labeled, covered with aluminum foil, and autoclaved for sterilization purposes. After autoclaving, the media was poured into Petri dishes which were left to cool and solidify. After solidification, the plates were packed and placed upside down in the refrigerator for storage and later use (Lammert and John, 2007).

The following media were used in this research study.

i. Mannitol Salt Agar (MSA)

Mannitol salt agar is the selective media and is used for the isolation and identification of *S. aureus* bacteria. The *S. aureus* colonies appeared yellow, having yellow zones, while, the Staphylococcus species showed red colonies having red zones. The agar was prepared by reconstituting the media powder in distilled water @111g/Liter.

ii. MacConkey Agar

MacConkey agar is the selective media and is used for the isolation of Gram-negative and enteric bacteria, *Escherichia* coli, which was selected for this study. The *E. coli* are lactose fermenter bacteria, decreasing the pH of the agar. Due to decreased pH, the neutral red MacConkey agar was absorbed by the bacteria and produced bright pink to red colonies on the agar. The *E. coli* colonies appeared pink to red, gave a doughnut appearance, and were encircled with a dark pink area due to the precipitation of bile salts. The MacConkey agar was prepared by dissolving the powder in distilled water at @50g /L.

iii. Nutrient Agar

Nutrient agar is a general-purpose growth media and was used for the growth of Streptococcus bacteria, which were identified by cultural and microscopic methods. The Streptococcus grew as yellow-colored colonies on the media and microscopically the bacteria appeared as chains (Esha and Anuradha, 2019). The bacteria can also be grown on the blood agar. The agar is prepared by dissolving the powder in distilled water @ 28 g/L (Figure 3).

Figure 3: The selective agar media used for the bacterial culture in the *in vitro* tests

iv. Broth Dilution Test

It is the earliest microbiological test, also known as the macro broth or tube dilution method. This method was adapted before the disk diffusion technique to determine the antibiotic potential of the Artemisinin extract by preparing its dilutions in the liquid growth media e.g. MSA. Three dilutions were prepared *i.e.* 1 mg/ml, 2 mg/ml and 5 mg/ml from the Artemisinin. Then 05 test tubes A, B, C, D, and E were prepared having broth in these tubes and bacterial inoculums from the cultures were introduced into the 04 test tubes, A, B, C and D, while, broth in the 5th tube "E" served as negative control. Then Artemisinin dilutions were added as 1mg/ml, 2mg/ml and 5mg/ml in test tubes A, B and C, respectively, while, D served as positive control having inoculums only and E served as negative control (without inoculums and Artemisinin extracts). After overnight incubation at 37 ^{0}C, tubes were examined for identification of the bacterial colonies or growths, showing the turbidity and the lowest dilution concentration of the Artemisinin extracts that prevented the growth of bacteria in the broth (Figure 4), which was termed as Minimum Inhibitory Concentration or MIC (Reller et al., 2009).

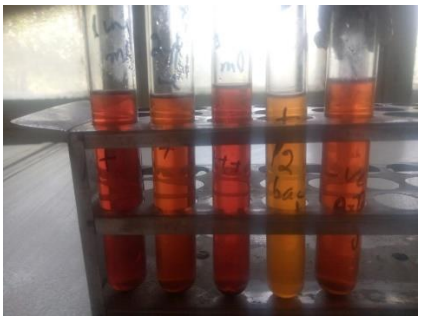

Figure 4: Broth dilution test in the MSA broth

v. Kirby Bauer Disk Diffusion Technique

The bacterial inoculums acquired from cultures were applied to MHA (Muller-Hinton Agar) plates of larger surface area, by streaking method of the sterile cotton swab. The plates were allowed to dry for 5 minutes. Then the Artemisinin impregnated disks along with the commercial disks were placed on the inoculated agar, using thumb forceps (Figure 5). After 24-48 hours of incubation of Petri dishes at 37 ^{0}C, the zones of growth inhibition were measured around each disk up to the nearest millimeter values. The zone diameters of these disks were interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI). The qualitative results of the disk diffusion test were recorded as S (susceptible), I (intermediate/moderate), or R (resistant) rather than MIC values (Bauer et al., 1966; Reller et al., 2009).

vi. In Vivo Evaluation Of Artemisinin

For *in vivo* test, 20 rabbits of the same age and breed were procured and divided into five groups *viz* Group A, B,

C, D and E. After an acclimatization period of 02 weeks, the rabbit models of each group were challenged with the *Staphylococcus aureus* and Streptococcus bacterial infection through intra-dermal inoculation route. For this purpose, the bacterial colonies (standard is 300 CFU/colony forming units) were diluted in 0.1ml Phosphate Buffer Saline (PBS) (Silvestre et al., 2020).

Figure 5: Kirby Bauer disk diffusion method and placing the antibiotic discs on the MHA

The *Escherichia coli* were injected intra-peritoneally into the rabbits @1.0 ml of *E. coli* suspension (@10^8 CFU/ml) and rabbit models were thus infected (Guo et al., 2017).

Rabbits of Group E (negative control) were administered with 1.00 mL phosphate buffer saline instead of bacteria. The group allocation of the experimental rabbits is given in Table 1.

Then the rabbits were treated using different concentrations of Artemisinin @1 mg/ml, 2 mg/ml and 5 mg/ml, respectively, via the intramuscular route, Group D served as positive control and was treated using commercial antibiotics e.g. amoxicillin @5 mg/kg body weight for 05 days, while, Group E served as negative control (not infected with bacteria) and treated using a placebo (1 ml phosphate buffer saline) through the intramuscular route (Figure 6).

RESULTS & DISCUSSION

Samples Cultured

To evaluate the antibacterial potential of the herb *Artemisia annua*, Artemisinin was extracted from this herb and was comparatively studied with the commercially available antibiotics, *in vitro* as well as *in vivo.*

To isolate the pure cultures of the bacteria, subcultures were prepared from all the growths on their respective media (Figure 7). And then the selected plates were streaked on the MHA (Merck, Germany) for a culture sensitivity test and comparison of the test herb, the Artemisinin extracts with the commercially available antibiotics (Figure 8).

Broth Dilution Test

The samples cultured were subjected to the broth dilution test, for the determination of the antibiotic potential of the Artemisinin extracts by preparing its dilutions in the MSA broth.

Table 1: Group allocation of the experimental rabbits in the trial.

Group Name	No. of Animals	Infected/ Not Infected	Treatment
Group A	4	Infected	Artemisinin @1mg/ml
Group B	4	Infected	Artemisinin @2mg/ml
Group C	4	Infected	Artemisinin @5mg/ml
Group D	4	Infected	Commercial antibiotic
Group E	4	Not Infected	Placebo (PBS)

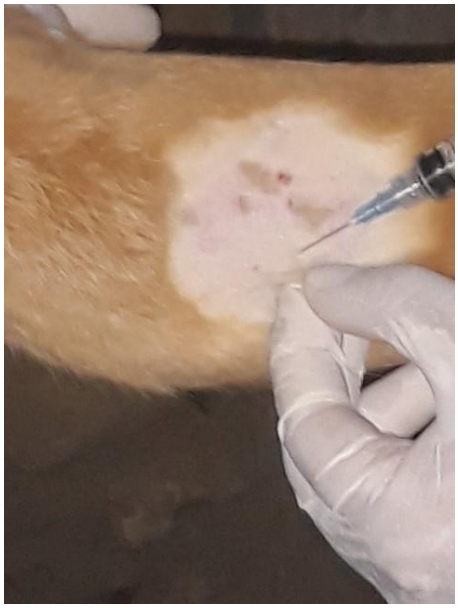
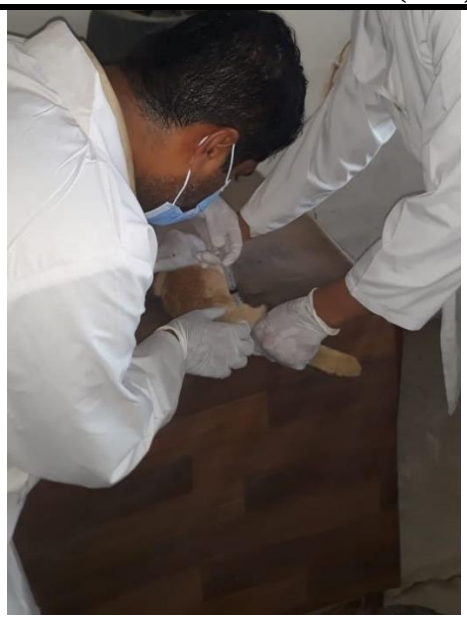

Figure 6: Inoculation of bacteria in the rabbits via subcutaneous route and subsequently their treatment.

For this purpose, three dilutions of 1 mg/ml, 2 mg/ml and 5 mg/ml were prepared from the Artemisinin extracts. And 05 test tubes A, B, C, D, and E were prepared having broth in these tubes and bacterial inoculums from the cultures were introduced into the 04 test tubes, A, B, C and D, while, the 5th test tube E served as negative control. Subsequently, the Artemisinin dilutions were added as 1 mg/ml, 2 mg/ml and 5 mg/ml in test tubes A, B and C, respectively, while, D served as positive control having inoculums only and E served as negative control (without inoculums and Artemisinin extracts). After overnight incubation at 37 ^{0}C, test tubes were examined for bacterial colonies and growths and it was manifested that test tube B was completely turbid, being positive control, and Test tube C having 5 mg/ml of Artemisinin extracts was clear followed by Test tube B and A (Figure 4).

Table 2: Details of samples cultured on different media used in the study.

Culture Media	Samples Cultured	Growth of bacteria in media	Name of Bacteria	Specimen type
Mannitol Salt Agar	40	28	*Staphylococcus aureus*	Milk (40)
MacConkey Agar	30	13	*Escherichia coli*	Intestinal contents of poultry (8/15), fecal material (5/15)
Nutrient Agar	30	21	Streptococcus	Milk samples (15/20), Blood samples (6/10)
Total Samples	100	62	----	----

Figure 7: Growth of *S. aureus* bacteria on the Mannitol Salt Agar and microscopic view of the slide prepared from this culture.

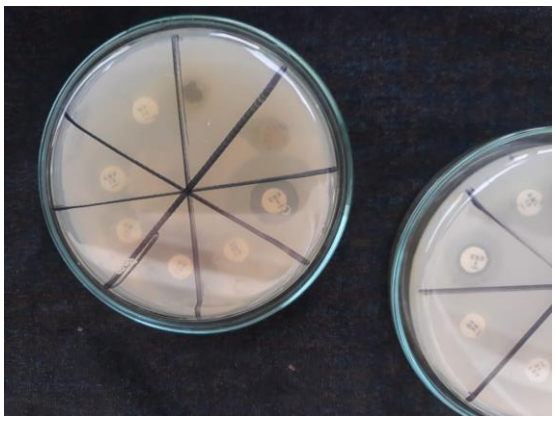

Figure 8: The culture sensitivity test on MHA and comparison of the zone of inhibition of different antibiotics.

The results revealed the significant antibacterial potential of the Artemisinin extracts @ 5 mg/ml, followed by 2 mg/ml and 1 mg/ml. So, the minimum inhibitory concentration of the Artemisinin extracts was found to be 1 mg/ml.

In Vitro Study of Antibacterial Potential of Artemisinin Extracts

To determine the antibacterial potential of Artemisinin extracts (*Artemisia annua) in vitro,* three bacterial species including *S. aureus, E. coli* and Streptococcus (Table 2) and the bacteriostatic zone of inhibition of Artemisinin extracts were studied against the test bacteria *Staphylococcus aureus,* Streptococcus and *Escherichia coli,* on the MHA media (Table 3) was also compared with the antibacterial potential of the commercially available antibiotics, by disk diffusion method, by measuring the zone of inhibition or bacteriostatic zone on the MHA (Figure 8) (Table 4).

The diameter of the zone of inhibition of the Artemisinin extracts was highly sensitive to *Staphylococcus aureus* (27.7 mm) followed by Streptococcus (22.3 mm) and moderate sensitivity to *Escherichia coli* (12.9 mm), as shown in Table 4. Thus Artemisinin showed a significant inhibitory effect against *S. aureus* and Streptococcus. Similar studies were conducted, which also confirmed the significant role of Artemisinin extracts against *S. aureus*. For instance, in a study, the methanol and ethanol extracts of the leaf extracts of Artemisinin were compared with commercial antibiotic ciprofloxacin. The results were also comparable to our study, in which the highest inhibition of the growth of *S. aureus* was observed with the Artemisinin extracts having a bacteriostatic zone of 16.5mm diameter (Tajehmiri et al., 2014). In another study, the water extracts of Artemisinin were compared with the commercial antibiotic ciprofloxacin, by disk diffusion method. The results revealed the highest sensitivity of *S. aureus* having a zone of inhibition of 21 mm, which was marked highly sensitive and supported our results. But the zone of inhibition against the *E. coli* was found at 15mm, which was also marked sensitive. It was concluded by the scientists that

Artemisinin extracts showed the highest sensitivity to *S. aureus* and weaker sensitivity to *E. coli*, as revealed in our results (Tao et al., 2020). In another study, the antimicrobial activity of Artemisinin and its precursors was tested against five bacterial species *viz S. aureus, Bacillus thuringiensis, B. subtilis, E. coli* and Salmonella. The study reported that Artemisinin was highly effective against *S. aureus* with TCs and had a significant inhibition zone of 3 ± 1.58 mm as that of the commercial antibiotic Streptomycin (Appalasamy et al., 2014). Rolta et al. (2021) also reported that the *Artemisia annua L.* herb can be strategically used as bio-enhancers against *S. aureus* and *E. coli* strains. Our study was also coinciding with the study conducted by Mohammed et al. (2022), whereby it was reported in the results that the crude extracts of the Artemisia herb showed significant antimicrobial potential against the *S. aureus* and *E. coli* pathogenic bacteria, with the diameter of the bacteriostatic zone as 20.33 and 15.67 mm, respectively.

Table 3: The bacteriostatic zones of inhibition of the Artemisinin extracts of *Artemisia annua* against different bacterial strains

Bacterial species	**Zone of Inhibition on MHA (mm)**										
	1	2	3	4	5	6	7	8	9	10	11
Staphylococcus aureus	25	27	23	29	28	27	28	26	27	28	27
Streptococcus	21	22	23	21	25	24	20	19	28	22	22
Escherichia coli	11	12	10	8	13	15	11	12	10	15	12

Note: Chi-squared test for given probabilities, X-squared = 93.431, df = 34, p-value = 1.89e-07

Table 4: The comparison of bacteriostatic zones of Artemisinin extracts with the commercial antibiotics on disc diffusion method against test microorganisms.

Bacterial species	**Zone of Inhibition on MHA (mm)**						
	Artemisinin	**Amox**	**Enro**	**Doxy**	**Tetra**	**Cipro**	**Oxy**
Staphylococcus aureus	27.7	25.1	21.1	17.3	19.3	30.4	29.4
Streptococcus	22.3	24.6	24.2	23.5	22.6	27.5	25.9
Escherichia coli	12.9	13.2	17.7	11.5	11.4	26.1	27.8

Note: Chi-squared test for given probabilities, X-squared = 60.655, df = 22, p-value = 1.787e-05.

Amox Amoxicillin; Enro* Enroflaxacin; Doxy* Doxycycline; Tetra* Tetracycline; Cipro* Ciprofloxacin; Oxy* Oxytetracycline.*

Table 5: Sensitivity of Artemisinin extracts and commercial antibiotics against the test microbes

Bacterial species	**Sensitivity against the test microbes**						
	Artemisinin	**Amox**	**Enro**	**Doxy**	**Tetra**	**Cipro**	**Oxy**
Staphylococcus aureus	Sensitive	Sensitive	Sensitive	Moderate	Moderate	Sensitive	Sensitive
Streptococcus	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
Escherichia coli	Moderate	Moderate	Moderate	Resistant	Resistant	Sensitive	Sensitive

Zone of Inhibition of Test Herb

From the current study, it was revealed that Artemisinin showed significant sensitivity to the test microbes *S. aureus* and Streptococcus, while, moderate sensitivity was observed against *Escherichia coli* (Table 5).

In vivo study of antibacterial potential of Artemisinin extracts of *Artemisia annua* in rabbit models

For *in vivo* study, a total of 20 rabbits of the same age and breed were allocated into 05 groups *viz* Group A, B, C, D and E (Table 1) and were initially infected with the *S. aureus* and then Streptococcus bacterial infection through intradermal inoculation route @ 300 CFU diluted in 0.1ml phosphate buffer saline and finally *Escherichia coli* were injected intraperitoneally into the rabbits @ 1.0 ml bacterial suspension of *E. coli* (@ 10^8 CFU/ml), with breach of 1-2 weeks posts each infection Thereafter, the infected rabbits showed typical clinical manifestations in terms of depression, dullness, inactivity, inappetence, anorexia, pyrexia, *etc* and the rabbits infected with *E. coli* were also diarrheic (Guo et al., 2017).

Then the rabbits were treated using different concentrations of Artemisinin @ 1 mg/ml, 2 mg/ml and 5 mg/ml, respectively, via the intramuscular route, Group D served as positive control and was treated using commercial antibiotics e.g. amoxicillin @ 5 mg/kg body weight for 05 days, while, Group E served as negative control and treated using a placebo (1 ml Phosphate buffer saline solution) through intramuscular route (Table 1). After 3-5 days of each infection, the following results were seen during this *in vivo* phase (Table 6). The results of our study revealed that all the rabbits infected with *S. aureus* recovered by treatment with the Artemisinin extract @ 1 mg/ml, 2 mg/ml and 5 mg/ml, but 03 out of 15 rabbits of Group A, B, C treated with Artemisinin extracts @ 1 mg/ml, 2 mg/ml and 5 mg/ml, had died.

Table 6: Treatment of rabbit models, via *in vivo* test against the test bacteria.

Treatment of rabbit models infected with *Staphylococcus bacteria*			
Group Name	No. of Animals	Treatment	Results
Group A	4	Artemisinin @1mg/ml	Recovered
Group B	4	Artemisinin @2mg/ml	Recovered
Group C	4	Artemisinin @5mg/ml	Recovered
Group D	4	Amoxycillin @5mg/kg body weight	Recovered
Group E	4	Placebo (PBS)	Recovered
Treatment of rabbit models infected with Streptococcus			
Group A	4	Artemisinin @1mg/ml	Recovered
Group B	4	Artemisinin @2mg/ml	Recovered
Group C	4	Artemisinin @5mg/ml	Recovered
Group D	4	Amoxycillin @5mg/kg body weight	Recovered
Group E	4	Placebo (PBS)	Recovered
Treatment of rabbit models infected with *Escherichia coli*			
Group A	4	Artemisinin @1mg/ml	2 recovered and 2 died
Group B	4	Artemisinin @2mg/ml	3 recovered and 1 died
Group C	4	Artemisinin @5mg/ml	Recovered
Group D	4	Ciprofloxacin @20mg/kg body weight	Recovered
Group E	4	Placebo (PBS)	Recovered

Therefore, as per the outcomes of this study, the Artemisinin extracts are highly effective against *S. aureus* and Streptococcal infections but have little effect on *in vivo* treatment of *E. coli* infections. Our results are supported by the study in which the Artemisinin extract was recommended against *S. aureus* and Streptococci bacteria (Tao et al., 2020). Another study also supported our results in which it was reported that the *Artemisia annua* herb had potential bactericidal peptide activities against the pathogenic bacteria in the rabbit models (Ding et al., 2019).

CONCLUSION

It has been concluded from the study that the Artemisinin extracts of the herb *Artemisia annua*, possessed significant antibacterial potential against the *Staphylococcus aureus* as well as Streptococcus bacteria, which was tested both *in vitro* and *in vivo*, using disk diffusion method and rabbit models, respectively. While Artemisinin extract had little effect against the *Escherichia coli* bacteria in both *in vitro* and *in vivo* trials. Therefore, as per findings of this study, the Artemisinin extracts are highly recommended for the treatment of bacterial infections caused by *S. aureus* and Streptococcus bacteria in animals and humans, as a complementary and alternative medicine (CAM), being a natural herb, bearing significant therapeutic potential with less adverse effects.

AUTHORS' CONTRIBUTION

All the authors contributed equally in this research trial.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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